AUSTRALIA

SAH 4. 8-8-01

Patent Office
Canberra

I, JONNE YABSLEY, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ5714 for a patent by GRADIPORE LIMITED filed on 18 February 2000.

WITNESS my hand this
Fourteenth day of February 2001

J R Yabsley

JONNE YABSLEY
ACTING TEAM LEADER
EXAMINATION SUPPORT & SALES

AUSTRALIA

Patents Act 1990

Gradipore Limited

PROVISIONAL SPECIFICATION

Invention Title:

Improved electrophoresis gels

The invention is described in the following statement:

Technical Field

This invention relates to the field of gel electrophoresis, particularly to pre-cast polyacrylamide gels having extended shelf-life.

Background Art

Gel electrophoresis is an important analytical or separation technique in which charged molecules are separated under the influence of an electric field with a gel being used as the support matrix. This technique is particularly suitable for the separation of biological macromolecules. The gels commonly used in this technique are composed of polyacrylamide or agarose. Polyacrylamide gels are used particularly for the separation of biomolecules such as proteins, peptides, DNA, RNA, lipids, carbohydrates, and recombinant proteins and the like, either naturally occurring or synthetic in which the acrylamide is used in slab form being precast prior to use. Traditionally, polyacrylamide gels were prepared individually prior to use by polymerising an acrylamide/cross-linker solution in a gel casting cassette to form a slab. Following separation of biomolecules, the gels are removed from the cassette, stained and/or dried so that the separated biomolecules may be visualised. Conventional polyacrylamide gels have the disadvantage of being relatively unstable and have a limited shelf-life.

As gels are often prepared on an individual basis prior to use, there can be variations between gels that have been cast separately such that direct comparison between separations using different gels are not reliable. Furthermore, the monomer components in polyacrylamide gels are relatively toxic and continued preparation of gels increases the potential of exposure of these toxic monomers to the operator. There has now been a move to the commercial preparation of pre-cast gels under controlled conditions. Operators now purchase pre-cast gels which have consistent and stable characteristics between batches. Unfortunately, pre-cast commercial gels still have the problem of having limited shelf-life and must be used within a given period of time to ensure accurate and reliable separations.

The current greatest limitation in the production and sale of pre-cast electrophoresis gels is the relatively short shelf-life, usually up to three months. This has been thought to be due to the hydrolysis of the amide groups in polyacrylamide to the carboxylic acid derivative in alkaline conditions. This hydrolysis is seen in the gels as loss of resolution of

separated material, change in migration of separated material and loss of bands.

Typically, gels are prepared using alkaline buffers and run under alkaline conditions, usually around pH 8.5. A buffer system using 2-Amino-2-hydroxymethyl-1,3-propanediol and hydrochloric acid (Tris-HCl) is a buffer of choice for "standard" polyacrylamide electrophoresis gels in denaturing conditions. These alkaline conditions are required to ensure that the biomolecules to be separated will be charged and hence will migrate under the influence of an applied electric potential. Although loss of stability in polyacrylamide gels occurs faster in alkaline conditions, this was thought to be a necessary disadvantage in standard gels including pre-cast gels. If the pH could be lowered closer to neutral, the hydrolysis or breakdown of the gel should be greatly reduced and so the gels will remain stable and useful for a longer period of time. Unfortunately, it has been difficult to find buffer systems that are compatible with a polyacrylamide medium that can be used around neutral pH's, that are suitable for gel electrophoresis and still provide a longer shelf-life for the gel.

Several companies have attempted to provide or develop gels having greater stability by using special buffer systems as it was believed that the standard Tris-HCl buffer system could not be changed significantly without adversely effecting separation efficiency. US Patent 3948743 (Bio-Rad Laboratories, 1976) discloses use of a strongly ionisable neutral salt in a concentration of 0.0005N to 1.0N at a pH of between 6 and 8. The neutral salt is preferably ammonium sulfate. The gels are then 'pre-run' in the buffer that is desired for separation to remove the salt before application of the sample. It would appear, however, that this method has not been commercialised.

US 4415655 and US 4481094 (TechAmerica Group, Inc., 1983 and 1984) disclose the use of a salt of 2-amino-2-methyl-1,3-propanediol at a pH of 6,4 to 7.3 in combination with 2-amino-2-methyl-1,3-propanediol taurine as a electrolyte buffer at a pH of 8.0 to 10.0.

US 5578180 and US 5922185 (Novel Experimental Technology, 1996 and 1999) disclose gels containing a buffer comprised of a primary organic amine or substituted amine with a pK_a near neutrality, titrated with hydrochloric acid or acetic acid to a pH between 5.5 and 7.5. Preferably, the primary organic amine or substituted amine is Bis-(2-hydroxyethyl)iminotris

(hydroxymethyl)methane (Bis-Tris). This system runs with the gel and buffer at a pH around neutral. The cathode buffer being a solution of a zwitterionic buffer, titrated to between pH 5.5 to 7.5 with sodium hydroxide or an organic base and the anode buffer being Tris(hydroxy methyl)amino-methane.

US 5464516 (Hymo Corporation, Atto Corporation, 1995) discloses gels which contain a buffer comprised of an acid, amine and an ampholyte that has the same number of anionic and cationic groups in each single molecule. The pH of these gels is between 4 and 7.5 which have been designed to give a wide separation range and stability. Of interest for the present invention is that the patent specification mentions that lowering the pH and Tris concentration in conventional gel systems does not allow sufficient movement of proteins.

Schagger and Von Jagow (Analytical Biochemistry 1987, 166: 368-379) developed a system for the separation of peptides and small proteins using a Tris-acetate buffer system. This system operates around pH neutral but it is more suited to being able to separate small proteins.

The present inventors have now surprisingly found that by manipulating the conventional Tris-HCl buffer system, stable gels can be prepared that have comparable separation characteristics as standard gels but having the advantage of long shelf-life.

Disclosure of Invention

In a first aspect, the present invention consists in a pre-cast polyacrylamide gel utilising a Tris-HCl buffer system and having a shelf-life of at least six months after storage at about 4^0C, wherein the shelf-life is determined by the gel producing an acceptable protein separation migration pattern and physical and resolution properties under electrophoresis conditions.

Preferably, the buffer system utilises a Tris concentration of about 0.15 to 0.25 M and a pH of between about 6.5 and 8.0. More preferably, the gel contains Tris at about 0.20 M with a pH of about 7.0.

The gels produced according to the present invention are substantially stable to polyacrylamide hydrolysis during storage for at least nine months.

Preferably, the gels have a shelf-life of about 12 months.

In a second aspect, the present invention consists in a method of preparing a pre-cast polyacrylamide gel, the method comprising polymerising

acrylamide in the presence of a cross-linking agent, water, a buffer system for the polyacrylamide gel and a polymerisation means;

wherein the buffer system comprises Tris-HCl and having a pH of between about 6.5 to 8.0; and

wherein the pre-cast gel has a shelf-life of at least six months after storage at about 4^{0}C as determined by the gel producing an acceptable protein separation migration pattern and physical and resolution properties under electrophoresis conditions.

Preferably, the buffer system utilises a Tris concentration of about 0.15 to 0.25 M and a pH of between about 6.5 and 8.0. More preferably, the gel contains Tris at about 0.20 M with a pH of about 7.0.

Preferably, the gels have a shelf-life of about 12 months.

Preferably, the polymerisation means is by redox type initiator using ammonium persulphate (APS) and N,N,N',N'-tetramethylethelenediamine (TEMED). Other free-radical initiator systems suitable for polymerising acrylamide gels including redox, thermal, photoactivation systems would also be suitable for the present invention.

In a third aspect, the present invention consists in an apparatus for use in gel electrophoresis, the apparatus comprising a slab of pre-cast gel according to the first aspect of the present invention adapted to be inserted in an electrophoresis apparatus.

In a fourth aspect, the present invention consists in a method of separating a biomolecule, the method comprising:

(a) applying the biomolecule to be separated to an electrophoresis apparatus according to the third aspect of the present invention;

(b) subjecting the gel to an electric field for sufficient time such that the biomolecule is caused to move into the gel.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1 shows separation of standard proteins in gels produced according to the present invention stored under accelerated storage conditions over various periods.

Figure 2 shows separation of standard proteins in gels produced by standard methods stored under accelerated storage conditions over various periods.

Figure 3 shows separation of standard proteins in gels produced according to the present invention stored over various periods.

Figure 4 shows separation of standard proteins in gels produced by standard methods stored over various periods.

Modes for Carrying Out the Invention

The approach of this work has been to stabilise Tris-HCl buffered gels by reducing the pH to around neutral conditions, usually near pH 7 by a reducing the concentration of Tris and the addition of further HCl. This approach has been taken as the present inventors have found that lowering the pH but not Tris concentration led to an unsatisfactory separation pattern despite a more stable gel (as compared with standard alkaline gels) being formed.

The present invention involves the use of the standard method of producing polyacrylamide electrophoresis gels where the concentration of hydrochloric acid is higher and thus the pH is lower. This means that as the polyacrylamide is no longer under alkaline conditions, the gels have a longer storage or shelf-life.

As a result in the changes in the buffer pH and concentration, the gels have a higher conductivity than conventional gels using Tris/HCl buffer system. Higher voltages are thus needed to move biomolecules through the gels at a faster rate. The inventors have also found that polyacrylamide gels produced according to the present invention are more efficient at separating larger sized proteins where other approaches to produce long-life gels have been more efficient for separating smaller proteins.

Accelerated storage studies show that the gels perform as desired as the migration values of the proteins in the gels were quite stable and the resolution was also quite good compared to a gel from the current range.

METHODS AND RESULTS

Gel production

Polyacrylamide gels were made using a conventional method used by the art. Acrylamide was mixed with a cross-linker (methylene bisacrylamide) and a solution of Tris such that the concentration of Tris in the final solution was 0.2 M. The solution was adjusted with HCl such that the pH of the solution was 7.0. Initiation of cross-linking was achieved by conventional method of redox initiation with ammonium persulphate (APS) and N,N,N',N'-tetramethylethelenediamine (TEMED).

Acrylamide concentrations

A typical example of the acrylamide concentrations in these gels was a stacking gel comprised of 5%T/4%C and a resolving gel of 10%T/3%C. Any conventional acrylamide concentrations, however, can be used in the present invention.

Running conditions

The gels were electrophoresed in 0.192 M Tris-glycine running buffer with 1% sodium dodecyl sulphate (SDS). The running voltage was 200 V for a time period of 90 minutes. Samples of snow pea protein dissolved in water and SDS sample buffer (Tris, HCl, glycerol, SDS, Bromophenol Blue) were used. Sigma Wide Range Molecular Weight marker was also used. The proteins in this marker were used to analyse the migration of protein through the gel. The proteins and their molecular weights are shown in Table 1.

Tris concentration range

It was determined from a series of experiments that the optimum conditions were 0.20 M Tris at pH 7.0. This was determined by manufacturing gels at pH 7.0 at various Tris concentrations. The concentrations of Tris tested varied in the range 0.375 M to 0.100 M. This range was chosen as 0.375 M is the conventional concentration used in polyacrylamide gels. In gels with this higher concentration of Tris (0.375 M) but having a lower pH than that used for standard gels, the proteins migrated too slowly through the gel. Also, the proteins did not achieve the equivalent separation pattern to gels at usual alkaline pH. This was expected and has been predicted by other research. This meant that lower concentrations of Tris were tested to see if the migration distances could be increased. Below concentrations of 0.150 M tested, the capacity of the Tris to buffer the solutions was reduced such that an unacceptable running pattern is observed

in the gel. It was found that a concentration of around 0.20 M gave a very good result. The range of Tris concentration that can be used with these neutral pH gels is preferably about 0.15 M to 0.25 M.

pH range

The range of pH values which had some stability in this system was 6.5 to 8.0. A number of pH values in this range were tested within the optimum Tris concentration of 0.200 M. Stability was found to be increased as the pH value was lowered. A gel with 0.2 M Tris concentration at pH 7.0 had an increase in stability of 50%.

Stability Results

Accelerated stability trials showed that gels produced by the present invention were more stable than conventional gels. The test gels were stored at 37°C and sampled each day. The migration values of specific proteins were determined and changes assessed over time.

The gels tested were outstanding in the stability of the migration values of the proteins in the gel. Figures 1 and 2 show a neutral pH gel with a Tris concentration of 0.2 M (Figure 1) compared to a conventional gel (Figure 2) in an accelerated stability trial (37°C storage). The results in Figure 1 shows that the protein migration values do not substantially change with time in gels made according to the present invention. The gels also did not suffer from loss of bands or loss of resolution/band sharpness. The accelerated stability trials showed that the new gels lasted longer than the control gels which were also stored at the elevated temperature. The distinctive results found in accelerated studies indicate that gel stability will be sustained during storage at conventional storage temperatures (around 4°C).

Further stability experiments using real time (4°C storage) of gels manufactured according to the present invention confirmed the findings of the accelerated studies. Gels having a pH of 8 and lower Tris concentration showed that the stability was in fact lengthened by approximately 50% when stored at 4°C for extended periods. Figure 3 shows the change in protein migration in gels produced according to the present invention for various times over six month storage. Figure 4 shows the change in protein migration in conventional gels stored at various times over six months. As can be clearly seen from the Figures, the migration patterns of a number of proteins did not substantially change in the gels produced according to the

present invention over the storage time tested. In contrast, gels produced having higher alkaline pH showed a decrease in performance over the six month test period. It can be seen from these results that a longer shelf-life was achieved in gels with a lower pH and a reduced Tris concentration produced according to the present invention.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

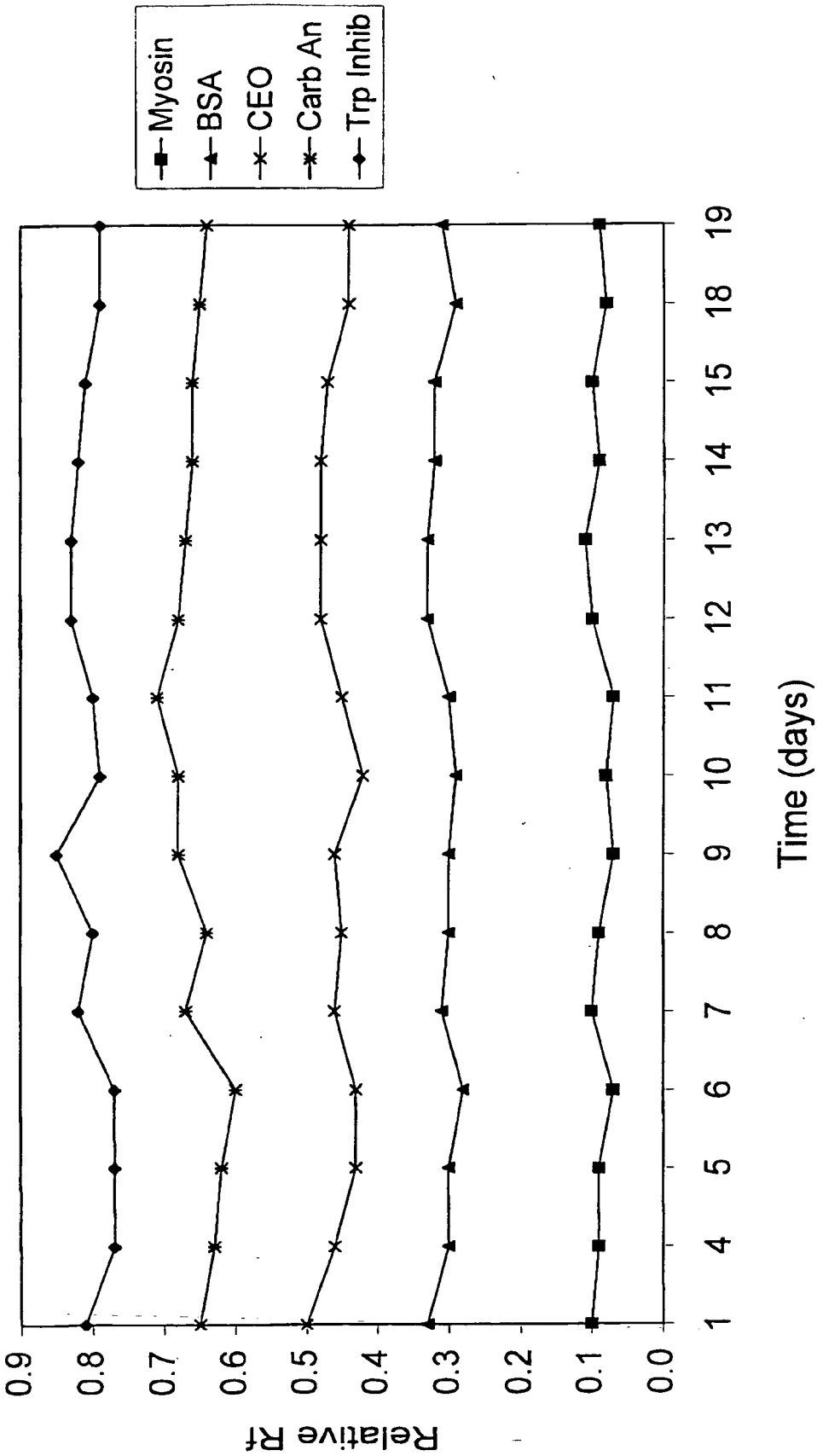
Dated this eighteenth day of February 2000

Gradipore Limited
Patent Attorneys for the Applicant:

AUSTRALIAN PATENT OFFICE REG 22.10 (2)

~~F B RICE & CO~~
ALLENS PATENT & TRADE MARK SERVICES

Figure 1


Myosin
BSA
CEO
Carb An
Trp Inhib
Relative Rf
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
4
5
6
7
8
9
10
11
12
13
14
15
18
19
Time (days)

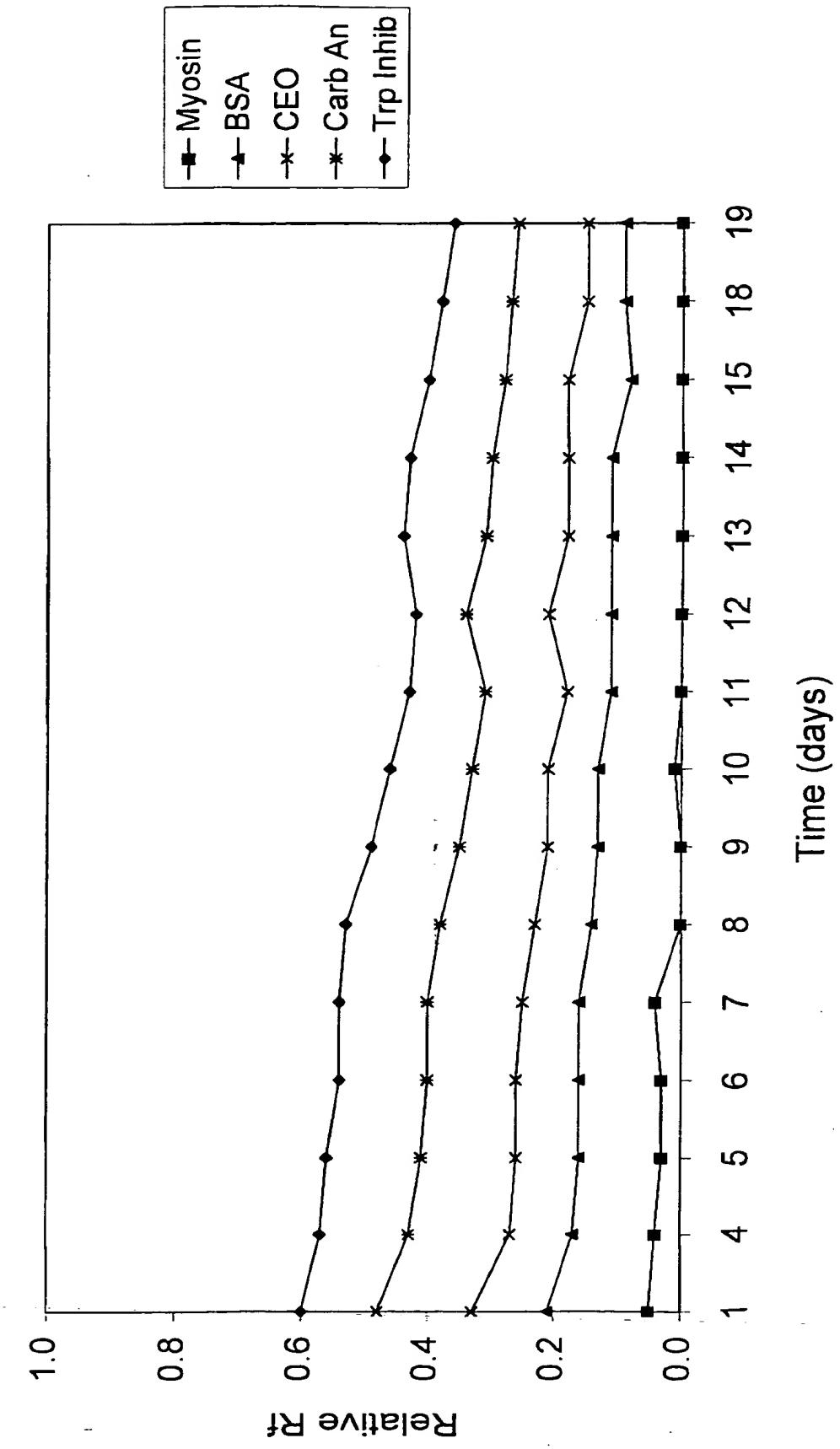

Myosin
BSA
CEO
Carb An
Trp Inhib
Relative Rf
1.0
0.8
0.6
0.4
0.2
0.0
1
4
5
6
7
8
9
10
11
12
13
14
15
18
19
Time (days)

Figure 2

Figure 3

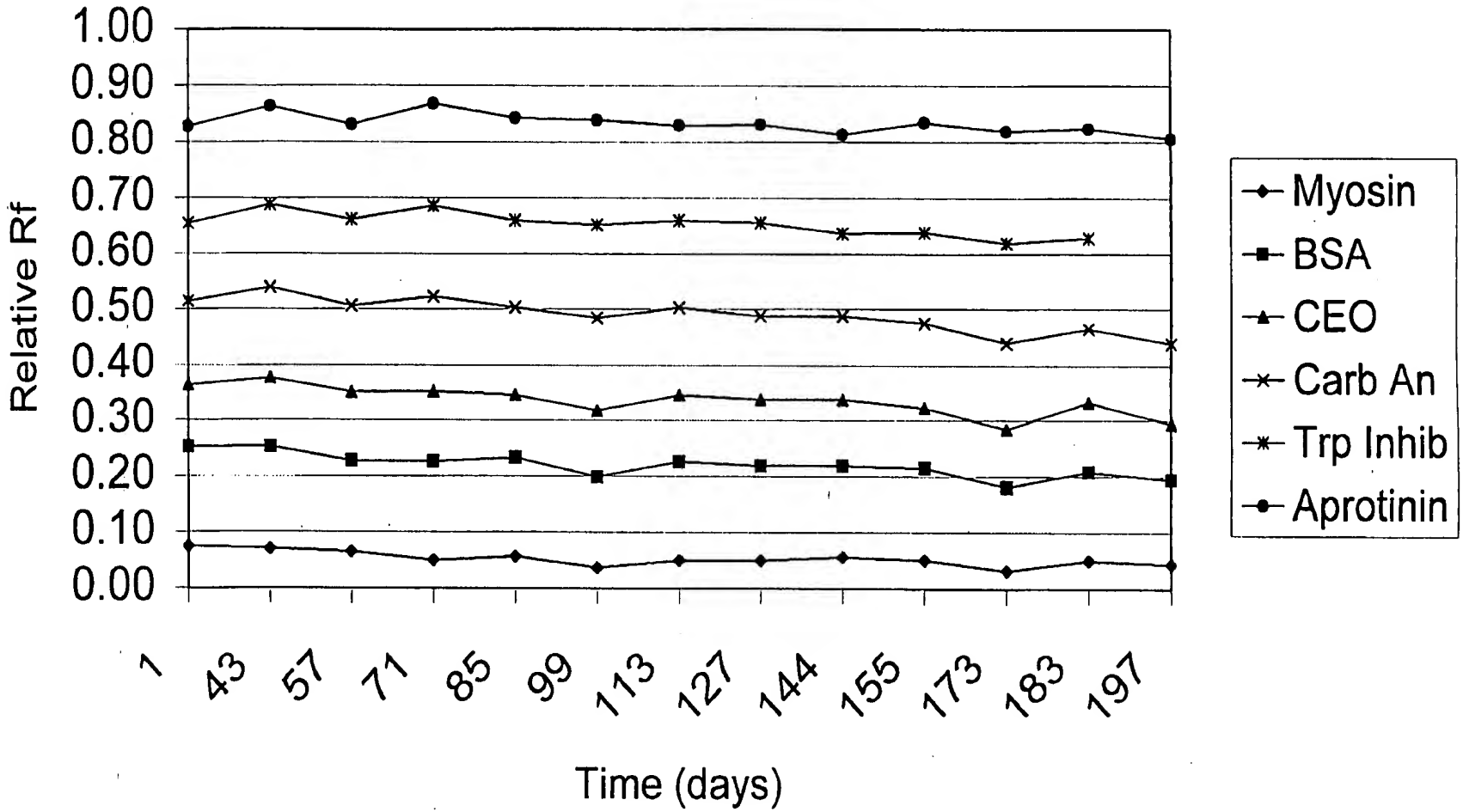
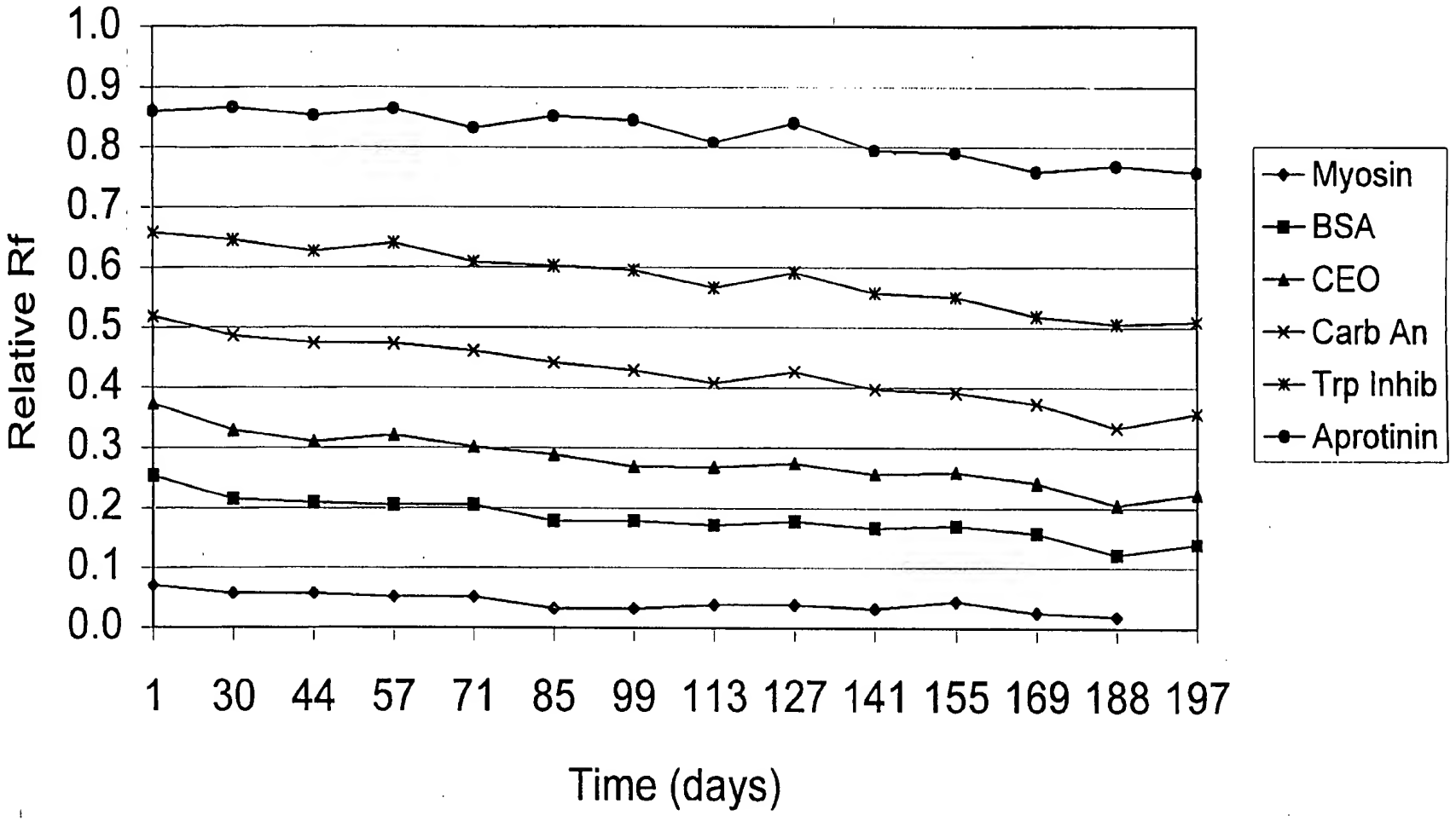

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Relative Rf
1
43
57
71
85
99
113
127
144
155
173
183
197
Time (days)
Myosin
BSA
CEO
Carb An
Trp Inhib
Aprotinin

Figure 4


Relative Rf
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
30
44
57
71
85
99
113
127
141
155
169
188
197
Time (days)
Myosin
BSA
CEO
Carb An
Trp Inhib
Aprotinin